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FINAL REPORT

We have investigated evolution-related aspects of bacterial rhodopsins, the unique retinal-based energy transducing systems of halophilic archae. The approach was to describe both structural and functional aspects: the *structure* by sequencing genes to explore which regions are conserved, and the *function* by comparing proton and chloride transport in the closely related systems, bacteriorhodopsin and halorhodopsin, respectively. In the latter, we have made a good start toward the ultimate goal of separating the attributes of the *general principles* of retinal-based ionic pumps from those of the *specific ion specificities*, by determining the thermodynamics of the internal steps of the protein-mediated active transport process (cf. point 1 below), as well as some of the intraprotein ion-transfer steps (cf. points 2 and 3 below). Our present emphasis is on continuing to acquire the tools for studying what distinguishes proton transport from chloride transport. We consider it important, therefore, that we have been able to provide firm mathematical grounds for the kinetics analyses which underlies these studies (cf. point 4 below). Our molecular biological studies have received a great boost from the expression vector for the *bop* gene based on a halobacterial plasmid, that we recently developed (cf. point 5 below). The highlights of this work are as follows (publications which resulted are in parentheses at the end of each topic).

1) Recent analysis of time-resolved difference spectra for bacteriorhodopsin in purple membranes yielded the general scheme $K \Leftrightarrow L \Leftrightarrow M_1 \rightarrow M_2 \Leftrightarrow N \Leftrightarrow O \rightarrow BR$ (Váró & Lanyi, *Biochemistry 29*, 2241-2250, 1990). Time-resolved difference spectra were now measured for Triton X-100 solubilized bacteriorhodopsin monomers as well. The results confirmed the derived kinetic scheme. The rate constants which involve proton release or uptake, i.e., k_{LM1}, k_{NO}, and k_{ON}, were significantly higher in the monomeric protein than in purple membrane; the other steps were less affected. Analysis of the temperature dependencies of the rate constants between 5°C and 30°C yielded the enthalpies and entropies of activation for all steps except the two absent back-reactions. Comparison of these with data for purple membranes (Váró & Lanyi, *Biochemistry*, *30*, 5016-5022, 1991) showed that the crystalline structure affects the energetics of the photocycle. In bacteriorhodopsin immobilized by the lattice of the purple membrane the entropy changes leading to all transition states were more positive. Thus, the forward reactions proceed with less conformational hindrance. However, the thermal (enthalpic) barriers are higher. These effects were particularly pronounced for the $M_1 \rightarrow M_2$ and $O \rightarrow BR$ reactions which are unique in that they are the only unidirectional reactions. Large changes of the enthalpy and entropy levels of intermediates in the M_2 - BR reaction segment, but not in the K - M_1 segment, upon solubilization of the protein are consistent with our earlier proposal that major protein conformational changes occur in the photocycle and they begin with the $M_1 \rightarrow M_2$ reaction. (cf. Váró & Lanyi, 1991 in list below).

2) During the M ~\ N \ BR reaction sequence in the bacteriorhodopsin photocycle proton is exchanged between asp96 and the Schiff base, and asp96 is reprotonated from the cyto-

plasmic surface. We probed these and other photocycle reactions with osmotically active solutes and perturbants and found that the M $\Leftrightarrow$ N reaction is specifically inhibited by withdrawing water from the protein. The N $\rightarrow$ BR reaction and the direct reprotonation of the Schiff base from the cytoplasmic surface in the recombinant asp96asn protein were much less affected. Thus, it appears that bound water is required inside the protein for reactions where a proton is separated from a buried electronegative group, but not for those where the rate limiting step is the capture of a proton at the protein surface. In the wild-type the largest part of the barrier to Schiff base reprotonation is the enthalpy of separating the proton from asp96, which amounts to about 40 kJ/mol. We suggest that in spite of this asp96 confers an overall kinetic advantage because when this residue becomes anionic in the N state its electric field near the cytoplasmic surface lowers the free energy barrier of the capture of a proton in the step that follows. In the asp96asn protein the barrier to the M $\rightarrow$ BR reaction is 20 kJ/mol higher than what would be expected from the rates of the M $\rightarrow$ N and N $\rightarrow$ BR partial reactions in the wild-type, presumably because this mechanism is not available. (cf. Cao et al. 1991 in list below).

3) The consequences of replacing asp85 with glutamate in bacteriorhodopsin were investigated. Similarly to the *in vitro* mutated and in *Escherichia coli* expressed protein, the chromophore was found to exist as a mixture of blue (absorption maximum 615 nm) and red (532 nm) forms, depending on the pH. However, we found two widely separated pK_as (about 5.4 and 10.4 without added salt), and argued that two blue and two red forms, in separate equilibria, exist. Both blue and red forms of the protein are in the 2-dimensional crystalline state. A single pK_a, such as in the *E. coli* expressed protein, was observed only after solubilization with detergent. The photocycle of the blue forms was determined at pH 4.0 with 610 nm photoexcitation, and that of the red forms at pH 10.5 and with 520 nm photoexcitation. The blue forms produced no M, but a K and an L like intermediate, whose spectra and kinetics resembled those of blue wild-type bacteriorhodopsin below pH 3. The red forms produced an early intermediate which is assumed to be K, as well as M and N. Only the red forms transported protons. Specific perturbation of the neighborhood of the Schiff base by the replacement of asp85 with glutamate was suggested by a) the shift and splitting of the pK_a for what is presumably the protonation of residue 85, b) a 36 nm blue-shift in the absorption of the all-*trans* red chromophore and a 25 nm red-shift of the 13-*cis* N chromophore, as compared to wild-type bacteriorhodopsin and its N intermediate, and c) significant acceleration of the deprotonation of the Schiff base at pH 7, but not its reprotonation and the following steps in the photocycle. (cf. Lanyi et al. 1992 in list below).

4) The bacteriorhodopsin photocycle contains more than five spectrally distinct intermediates; the complexity of their interconversions has precluded an *exact* solution of the kinetics. A representation of the photocycle of mutated asp96asn bacteriorhodopsin near neutral pH was given earlier (Váró & Lanyi, *Biochemistry 30*, 5008-5015, 1991) as BR $-\underline{h\nu}$ $\rightarrow$ K $\Leftrightarrow$ L $\Leftrightarrow$ M_1 $\rightarrow$ M_2 $\rightarrow$ BR. Three base spectra, obtained by averaging time-resolved difference spectra for this simpler system, were used to generate all allowed sets of spectra for K, L and M (i.e. M_1 + M_2). Invalid spectra were eliminated progressively in a grid-search, based on what is expected, empirically and from the theory of polyene excited states, for rhodopsin spectra. Significantly, the averaged spectra changed little after the

first and simplest of the search criteria, which disallowed negative absorptions and more than one maximum for the M intermediate. However, the number of solutions and the standard deviations decreased strongly after the additional filters using other, independent criteria. We concluded from the results that the solution array is distributed densely around a single unambiguous global minimum. Data at three different temperatures between 5 and 25°C confirmed the earlier derived spectra and model for this photocycle. (cf. Zimányi & Lanyi, 1992 in list below).

5) Until now the only available workable system for the expression of mutated *bop* (bacteriorhodopsin) genes was in *Escherichia coli*, and required *in vitro* reconstitution of the denatured polypeptide. This approach is technically difficult and raises questions about possible differences from the *in vivo* folded proteins. Using a newly described transformation system and a halobacterial plasmid vector, we have shown that it is possible to reintroduce the *bop* gene into bacteriorhodopsin-negative strains of *H. halobium*. The *bop*-carrying plasmid carries also a gene for mevinolin resistence for selection, and expresses native bacteriorhodopsin in wild-type like amounts. (cf. Ni et al. 1990 in list below). It has proved to be a suitable vector for producing site-specific mutants. More recent work (Needleman et al. 1991; Lanyi et al. 1992) has shown that, as suspected, at least some mutated bacteriorhodopsins expressed in *H. halobium* have different properties from that expressed in *E. coli*.

Publications credited to the NASA grant in the last funded competitive renewal period

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